Granulocyte Macrophage–Colony-Stimulating Factor (GM-CSF) Decreases CD1a Expression by Human Langerhans Cells and Increases Proliferation in the Mixed Epidermal Cell-Lymphocyte Reaction (MELR)

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Langerhans cells (LC) undergo a variety of phenotypic and functional changes in vitro. To determine the effects of granulocyte macrophage–colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and interleukin 1-α (IL-1) on LC phenotype in vitro, epidermal cell suspensions were enriched for LC by density-gradient centrifugation and cultured in the presence of 10 ng/ml of these cytokines. The percentage of cells expressing the surface protein CD1a was determined by flow cytometry. This percentage typically dropped after 48 h culture in both control and cytokine-treated medium to less than half that of the starting value. By the fifth day, the percentage of cells expressing CD1a in TNF-α and IL-1–treated cultures was still near half of the starting value, slightly above that of control cultures. Treatment with GM-CSF caused large and consistent decreases in the percentage of epidermal cells expressing CD1a. Cell viability in each of the three cytokine-treated cultures was identical to the control cultures, with essentially all cells having died by the sixth day after isolation. To determine the functional effects of these cytokines, the cytokine-containing medium was replaced after 72 h with medium containing purified allogeneic T cells and proliferation measured. Preliminary experiments showed no increased proliferation induced by IL-1 or TNF-α–treated epidermal cells. GM-CSF–treated epidermal cells induced 2–3 times more T-cell proliferation than epidermal cells cultured without additional cytokines. We conclude that GM-CSF, a cytokine known to be produced by keratinocytes in vitro, decreases CD1a expression by human LC and increases their ability to stimulate proliferation by allogeneic T cells. *J Invest Dermatol 95:359–362, 1990*

Human Langerhans cells (LC) are bone marrow–derived dendritic cells [1] that circulate to regional lymph nodes from the epidermis and are capable of inducing allogeneic, antigen specific, and cytotoxic T-cell proliferation [2–6]. LC express CD1a (T6) and class II major histocompatibility complex (MHC) antigens [5,7]. LC expression of class I MHC proteins is controversial; freshly isolated epidermal LC express little or no class I MHC protein [8–10], whereas grafted LC in murine bone marrow chimeras have been shown to express significant levels of class I MHC protein in the host epidermis [11]. Human LC quickly lose CD1a expression and increase class I and class II MHC molecule expression when removed from the epidermis and placed in culture [10,12].

Freshly isolated LC are poor stimulators of allogeneic T cells in the mixed epidermal cell-lymphocyte reaction (MELR), but become some of the most potent known stimulators of this reaction when maintained in culture for 2–3 d [6,10]. Studies in murine systems suggest that epidermally associated cytokines such as granulocyte macrophage–colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and interleukin 1-α (IL-1) may influence the development of this ability in vitro [13–15]. To determine how these cytokines affect the phenotype and function of human LC in vitro, we cultured purified LC from normal human skin with these cytokines and examined their expression of CD1a and their allogeneic stimulatory ability in the MELR.

MATERIALS AND METHODS

Isolation of Epidermal Cells Epidermal cells were separated as previously described [16]. Briefly, samples of normal skin were obtained from plastic surgery specimens. The skin was trimmed to the dermis and incubated overnight at 4°C, dermal side down, in Tyrodes solution with 10 mg/ml Dispase (Boehringer Mannheim, Indianapolis, IN). The epidermis was peeled off and cell suspensions were obtained by incubation in 0.25% trypsin (Gibco, Grand Island, NY) at 37°C for 15 min, followed by mechanical disruption by vortexing and filtering through three layers of gauze (USP type VII, Johnson and Johnson, New Brunswick, NJ). Viable cells were enriched by density-gradient centrifugation over Histopaque 1077

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Abbreviations:

GM-CSF: granulocyte macrophage–colony-stimulating factor
IL-1: interleukin 1-α
LC: Langerhans cells
MELR: mixed epidermal cell-lymphocyte reaction
MHC: major histocompatibility complex
PBS: phosphate-buffered saline
TNF-α: tumor necrosis factor-α

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(Sigma, St. Louis, MO) at 300 × *g* for 20 min at 4°C. Cells remaining at the interface were collected and viability assessed by trypan blue exclusion. This technique reliably yielded 97–100% living cells. As noted by Romani and colleagues [10], the percentage of CD1a+ cells was highly variable; in our hands a maximum of 30% of cells expressed CD1a, but we routinely found greater than 95% of the CD1a+ cells at the interface.

Immunofluorescence and Cytometry Cells were stained with the indirect technique as previously described [16]. Briefly, the cells were incubated with a mouse monoclonal reacting with the CD1a protein (produced by the ATCC hybridoma CRL 8020) at a final concentration of 10 μg/ml in 1 ml phosphate-buffered saline (PBS) and 5% calf serum (GIBCO) for 1 h at 4°C, pH 7.4. After washing twice with PBS and 5% calf serum, a second incubation was performed with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Becton-Dickinson, Mountain View, CA), 1 μg/10^6 cells, in 1 ml PBS with 5% calf serum for 1 h at 4°C. After 2 more PBS washes (this time with 1% calf serum), the percentage of cells expressing CD1a was determined on a Becton-Dickinson FACS-IV cell sorter. Controls included the omission of the primary antibody as well as irrelevant primary monoclonal antibody.

Langerhans Cell Cultures After determination of CD1a positivity, cells were plated out at 10^6 CD1a+ cells/ml in 12-well flat-bottom plates (Corning Glass Works, Corning, NY). The culture medium consisted of RPMI 1640 (GIBCO), 10% defined fetal bovine serum (HyClone Labs Inc., Logan, UT), and 1% penicillin G/streptomycin/amphotericin B (GIBCO). Recombinant human GM-CSF, IL-1 (gifts of Dr. Steven Gillis, Immunex Corp., Seattle, WA), or TNF-α (courtesy of Dr. Thomas Kupper) were added to the experimental cultures to a final concentration of 10 ng/ml. This concentration of cytokines was chosen to give a maximum effect after preliminary studies showed no difference between the effects of 50 or 10 ng/ml of each cytokine.

Preparation of T Cells Peripheral mononuclear cells were obtained from the blood of healthy volunteers by centrifugation through Histopaque as above. Cells were suspended in culture medium at a density of 10^7 cells/ml and monocytes allowed to adhere to a plastic culture flask (Corning Glass Works) for 3–12 h. Nonadherent cells (lymphocytes) were then removed and incubated for 1 h at room temperature with saturating amounts of an antibody that recognizes a framework determinant present on the class II MHC proteins HLA-Dr and HLA-Dp (produced by the IVA12 hybridoma, ATCC #HB 145). After washing, cells were then incubated for 30 min at 37°C with an iron-conjugated goat anti-mouse secondary antibody (Advanced Magnetics Inc., Cambridge, MA) and precipitated for 20 min to a magnetic plate (Advanced Magnetics Inc.). Nonmagnetic cells were then washed from the plate. This procedure was repeated and reliably yielded a cell suspension of 94–97% T cells, as assessed by expression of T-cell receptor–associated CD3 antigen by flow cytometry.

Mixed Epidermal Cell–Lymphocyte Cultures Epidermal cell suspensions enriched for LC were plated out in triplicate over a range of 0–3000 LC, 200 μl/well in 96-well round-bottomed plates (Corning Glass Works). Culture conditions and cytokine concentrations were as above. On day 3 of culture (72 h), the plates were centrifuged for 5 min at 300 × *g* and the medium replaced with the RPMI (GIBCO). Plates were centrifuged again and RPMI replaced with medium containing 10^5 purified allogeneic T cells but no added cytokines. These mixed epidermal cell–lymphocyte cultures were then incubated for 4 d more, pulsed with 1 μC of ^{3}H-thymidine (Amersham Inc., Arlington Heights, IL) per well, and harvested 16 h later, and the counts/min incorporated assessed with an LBK scintillation counter.

RESULTS

Expression of CD1a The culture conditions used were chosen to support T cell proliferation, not necessarily to grow LC. To assure that the results did not simply represent differential survival of LC in the different cultures, the percentage of viable cells was determined by trypan blue exclusion on days 0, 2, 3, and 5. Viability was virtually identical in control and cytokine-treated cultures, which showed steady cell death until day 6 of culture, by which time virtually all cells had died. In total, six experiments of the effects of cytokines on CD1a expression were carried out using skin specimens from six separate individuals. The pattern of CD1a expression by LC treated with cytokines versus control was similar in all six cases. Figure 1 shows the mean of two such cases that are readily comparable due to their high and similar levels of CD1a expression on day 0 (30% and 29.5%). Any given specimen yielded only enough LC for CD1a determinations on three days (day 0, day 5, and one intermediate day), and we have therefore combined two such experiments to better illustrate the time course of CD1a expression over the 6-d period of LC viability in vitro. By the second day of culture, the percentage of cells expressing CD1a dropped from 30% on isolation to 12.4% in medium alone (control group), 13.5% with GM-CSF, 13.0% with IL-1, and 11.0% with TNF-α. By day 5 of culture, CD1a expression among control cells dropped to 9.5%, whereas it was 13% in TNF-α–treated and 15% in IL-1–treated cells. Only 4.2% of GM-CSF–cultured cells were CD1a+ by day 5. The percentage of the cells treated with GM-CSF that expressed CD1a on day 5 of culture was less than half that of control cells or cells treated with IL-1 or TNF-α. Thus, the differences between the GM-CSF–treated cultures and the other cultures were large on day 5, and were statistically significant by a one-tailed Student *t* test, $p = 0.01$. As previously noted by Walsh and Seymour [17], cultures treated with IL-1 showed a statistically significant increase in the percentage of cells expressing CD1a compared to control cultures ($p = 0.02$ by a one-tailed Student t test). In our studies, however, the magnitude of the difference between IL-1–treated and control cultures was not great.

Allogeneic T-Cell Stimulation Preliminary experiments showed no increased stimulation by epidermal cells precultured in IL-1 or TNF-α, and the remainder of the experiments were done only with GM-CSF–treated and control cultures. Eight such experiments were performed using surgical specimens from eight different patients. Epidermal cells precultured for 72 h with GM-CSF were always more potent stimulators of allogeneic T-cell proliferation than cells cultured in medium alone. Figure 2 shows the results of triplicate cultures from a representative experiment using LC from one specimen and T cells from one donor. These data show increased allogeneic stimulation at all doses of LC tested, as well as a direct relationship between the number of LC in culture and the level of T-cell proliferation, regardless of the culture conditions

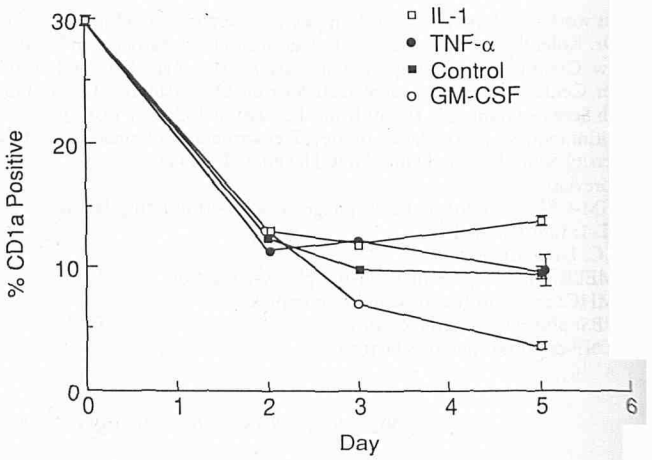

Figure 1. The percentage of epidermal cells retaining CD1a surface expression in cultures treated with cytokines compared to control (untreated) cultures. *Error bars,* SEM.

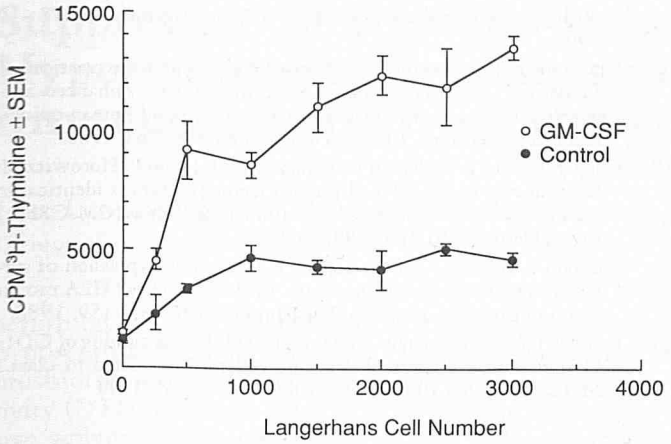

Figure 2. Allogeneic T-cell proliferation in response to cytokine treated or control epidermal cells. *Error bars,* SEM of triplicate cultures.

used. The differences between stimulation by GM-CSF–treated cells and control cells were significant at $p < 0.01$ at all doses of LC tested, except at the lowest dose, where $p = 0.05$ (one-tailed Student t test). This general pattern was seen in all our co-cultivation experiments. These results indicate that GM-CSF is augmenting LC ability to stimulate allogeneic reactions on a cell-by-cell basis. There was no significant difference in the level of T-cell proliferation observed in wells that contained GM-CSF or unaugmented medium but no LC, even though these wells were treated as if they did contain LC in the 72-h preculturing period. This observation precludes the possibility that the presence of contaminating GM-CSF alone is responsible for the enhanced T-cell proliferation seen in GM-CSF–treated cultures.

DISCUSSION

Most functional studies of human LC have involved crude epidermal cell suspensions. It is generally accepted that LC are the stimulating cells because selective lysis of LC ablates the proliferative response and, therefore, it is reasonable to assume that this remains true in cytokine-treated cultures [4,18]. Studies of murine and human epidermal cells have demonstrated that freshly isolated LC are poor stimulators of allogeneic T cells in the MELR [6,10]. When maintained in culture for 2–3 d, however, LC become potent allogeneic T-cell stimulators [6,10]. These functional changes are associated with phenotypic changes, such as increased expression of class I and class II MHC molecules by both human and murine LC, and loss of expression of CD1a by human LC [9,10,12]. Experiments with murine LC have also shown that GM-CSF may accelerate and TNF-α may retard these phenotypic and functional changes [13–15].

CD1a is a cell-surface glycoprotein that has many similarities to class I MHC molecules, is expressed in a distribution that is reciprocal to these proteins in vivo [7,20,21], and has no clearly defined functional homologue in mice. As a result, it has been argued that CD1a represents a novel type of class I MHC molecule, and as such may be involved in the presentation of antigen to T cells [21]. Study of class I MHC expression by human LC is technically difficult because keratinocytes also express these molecules. However, as GM-CSF increases expression of class I MHC molecules by murine LC in vitro, we reasoned that it might have a reciprocal effect on CD1a expression by human LC. Our studies support this hypothesis by showing that GM-CSF decreases CD1a expression by human LC. Although indirect, this finding also supports the hypothesis that GM-CSF has effects on human LC that are similar to its effects on murine LC.

More direct support for the latter hypothesis comes from our demonstration that GM-CSF–treated human epidermal cell suspensions have a markedly increased ability to stimulate proliferation of allogeneic T cells, similar to the effect of GM-CSF on murine LC. Because keratinocytes can produce GM-CSF [19], it is possible that the exogenous GM-CSF used in these experiments merely supplemented GM-CSF already present in the cultures, thus accelerating the phenotypic and functional changes. Although our experiments with TNF-α– and IL-1–treated epidermal cells showed that they were slightly less efficient stimulating cells than the control cells, the differences between these three groups were not large. As the effects of these cytokines on LC appear to be opposite to those of GM-CSF in murine systems, it is possible that endogenous GM-CSF obscured an effect in our studies.

It is not clear from our studies whether the observed effects of GM-CSF on LC are due to direct changes in LC physiology and phenotype or to the secondary production of other signalling molecules. Studies using antibodies that block the effects of GM-CSF and other epidermal cytokines are being planned to address this question. It is clear that the effects are not due to changes in LC viability, as viability was the same in all our experimental groups. In this way, our results differ from those in murine systems where GM-CSF and TNF-α appear to prolong LC viability in vitro [13,15]. However, because the culture conditions used were optimized for T-cell proliferation rather than for epidermal cell growth, this finding should not be taken as evidence that these two cytokines are incapable of promoting human LC growth. Our studies have shown that GM-CSF produces some effects in cultured human LC that parallel those seen in murine LC and thus support the concept that GM-CSF may have an important role in the immune regulation of human skin.

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